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Prognostic Significance of Immunoreactive Neutrophil Elastase in Human Breast Cancer: Long-Term Follow-Up Results in 313 Patients[1]

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Abstract

OBJECTIVE: **We have measured the concentration of immunoreactive neutrophil elastase (ir-NE) in the tumor extracts of 313 primary human breast cancers. Sufficient time has elapsed, and we are now ready to analyze its prognostic value in human breast cancer.** ***METHODS:*** **ir-NE concentration in tumor extracts was determined with an enzyme-linked immunosorbent assay that enables a rapid measurement of both free-form ir-NE and the α_1-protease inhibitor–complexed form of ir-NE. We analyzed the prognostic value of this enzyme in human breast cancer in univariate and multivariate analyses.** ***RESULTS:*** **Patients with breast cancer tissue containing a high concentration of ir-NE had poor survival compared to those with a low concentration of ir-NE at the cutoff point of 9.0 μg/100 mg protein (*P* = .0012), which had been previously determined in another group of 49 patients. Multivariate stepwise analysis selected lymph node status (*P* = .0004; relative risk = 1.46) and ir-NE concentration (*P* = .0013; relative risk = 1.43) as independent prognostic factors for recurrence.** ***CONCLUSIONS:*** **Tumor ir-NE concentration is an independent prognostic factor in patients with breast cancer who undergo curative surgery. This enzyme may play an active role in tumor progression that leads to metastasis in human breast cancer.**

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Introduction

During invasion and metastasis formation, tumor cells confront a variety of natural tissue barriers *in vivo*, such as basement membranes and surrounding tissue stromal matrices composed of elastins, collagens, and proteoglycans. It is thus necessary for tumor cells to elaborate a battery of extracellular matrix (ECM)–degradative enzymes to achieve metastatic invasion. Many different types of ECM-degradative enzymes have been implicated in the invasive growth and metastasis of cancer cells [1–3].

The production of tumor cell proteases, including collagenase [4,5], plasminogen activator [6,7], and cathepsin B [8], has been implicated in tumor cell invasion into adjacent tissues and metastasis. Another proteolytic enzyme thought to be involved in this process is elastase, which is the only protease that is able to degrade insoluble elastin—a structural component of elastic tissues such as blood vessel, skin, lung, and breast tissues.

There are three well-characterized mammalian elastases. The best characterized is porcine pancreatic elastase I, first described by Balo and Banga [9], which is a serine protease secreted in zymogen form by pancreatic acinar cells. The second class of mammalian elastase is neutrophil elastase (NE), the neutral protease found in granules of human polymorphonuclear leukocytes [10,11]. The third mammalian elastase is a metalloprotease, which is secreted by inflammatory macrophages [12]. Of these elastases, NE exhibits the most proteolytic activity under physiological conditions.

The presence of elastinolytic activities in human breast cancer tissue has been demonstrated by Hornebeck et al. [13]; however, in their study, it was not determined whether the activity could be attributed specifically to breast cancer cells. Thereafter, several investigators have described elastinolytic enzyme production by human and rodent mammary tumor cells [14–16], although these enzymes have not been isolated or characterized.

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In this connection, we previously have reported that NE is produced by human breast cancer cell lines, using a highly specific and sensitive enzyme immunoassay [17]. In addition, we conducted a prognostic study of 313 patients with breast cancer who underwent curative mastectomy and have reported a preliminary result suggesting that the concentration of immunoreactive neutrophil elastase (ir-NE) in tumor extracts may affect prognosis in human breast cancer [18]. Sufficient time has elapsed, and we are now ready to analyze prognosis in patients with breast cancer.

Materials and Methods

Patients

Three hundred thirteen patients with breast cancer in the present analysis constitute 100% of our previous study population [18]. These patients underwent curative mastectomy with lymph node dissection at the Department of Surgery II, Kumamoto University Hospital, between March 1982 and April 1989. The median follow-up period for patients was 18.5 years (range, 14.0–21.1 years). The clinicopathological characteristics of the 313 patients are summarized in Table 1.

Assay for ir-NE

Breast cancer specimens were homogenized and extracted with 50 mM Tris–HCl buffer (pH 7.4) containing 0.25% Triton X-100, as described previously [23]. The resulting supernatant was assayed for ir-NE concentration as described below.

The concentration of ir-NE in tumor extracts was determined with a newly established enzyme immunoassay kit (Mochida Pharmaceutical Co., Tokyo, Japan). This is a sensitive assay that enables a rapid measurement of both NE-complexed α_1-protease inhibitor (α_1-PI) and free-form NE [24]. When 0.1 ml of tissue extract was used, the detection limit of ir-NE was 0.063 μg/100 mg protein. The intra-assay and interassay coefficients of variation were 3.2% to 5.6% and 5.1% to 8.7%, respectively.

To measure the level of free-form and α_1-PI–complexed form in tissue extracts, we determined the concentration of ir-NE in all samples in the presence and in the absence of an excess amount (100 μg/ml) of α_1-antitrypsin (Sigma, St. Louis, MO) using the conventional Merck kit (E. Merck, Darmstadt, Germany) according to the method of Neumann et al. [25]. Because the Merck kit detects only NE complexed with α_1-PI, the difference between these concentrations was regarded as free-form ir-NE, and the concentration in the absence of α_1-antitrypsin was regarded as the α_1-PI–complexed form of ir-NE.

Survival Analysis

Routine postoperative follow-up consisted of clinical evaluations every month for the first 2 years and every 3 to 6 months thereafter. Disease recurrence was documented by physical examination, roentgenographic and laboratory tests, and other relevant diagnostic procedures. The major statistical endpoint of this study was disease recurrence (distant recurrences only) and was calculated from the day of operation to the day of discovery of recurrence or the last known date alive. Event time distribution was estimated with the method of Kaplan and Meier [26]. Differences between death distributions were tested for statistical significance with the log-rank test [27]. For simultaneous control of the effects of many variables on differences in death rates, a multivariate proportional hazards regression model [28] was used. $P < .05$ was considered significant.

Table 1. Relation between ir-NE Content in Tissue Extracts and Clinicopathological Factors of Human Breast Cancer (n = 313).

Characteristic Content (μg/100 mg Protein)	n (%)	ir-NE (Mean ± SD)
Menstrual status		
Premenopause/perimenopause	179 (57)	5.24 ± 4.11
Postmenopause	134 (43)	4.32 ± 3.50
Tumor size (cm)		
< 2.0	57 (18)	3.38 ± 1.13
2.0–5.0	176 (56)	4.55 ± 3.25
> 5.0	80 (26)	7.20 ± 5.12*
Lymph node status		
Node-negative	178 (57)	2.54 ± 1.90
Node-positive	135 (43)	5.47 ± 4.24†
Histologic type‡		
Papillotubular	72 (23)	3.26 ± 3.01
Solid–tubular	117 (37)	4.66 ± 3.86
Scirrhous	111 (35)	5.11 ± 3.92
Other	13 (4)	4.28 ± 3.99
Histologic grade§		
I	90 (29)	4.42 ± 3.76
II	122 (39)	4.63 ± 3.73
III	101 (32)	5.89 ± 5.01
Vessel involvement		
Absent	194 (62)	4.22 ± 4.05
Present	119 (38)	4.96 ± 3.97
Estrogen receptor¶		
Positive	159 (51)	5.52 ± 4.03
Negative	124 (40)	4.14 ± 3.63
Unknown	30 (10)	4.68 ± 4.00
Progesterone receptor¶		
Positive	94 (30)	5.38 ± 4.24
Negative	178 (57)	4.29 ± 3.62
Unknown	41 (13)	4.21 ± 3.97
Type of surgery#		
Halsted	75 (24)	4.51 ± 3.93
Modified	238 (76)	5.72 ± 4.10
Adjuvant therapy		
Endocrine therapy	75 (24)	3.45 ± 3.19
Chemotherapy	45 (14)	4.51 ± 4.13
Both	92 (29)	3.96 ± 3.22
None	101 (32)	4.07 ± 3.18
Immunoreactive NE		
< 9.0	261 (83)	
≥ 9.0	52 (17)	

*$P < .002$, compared with <2.0 and 2.0 to 5.0 cm.

†$P < .001$, compared with node-negative.

‡Breast tumor was analyzed according to the classification of the Japanese Breast Cancer Society [19]. When histologic typing was performed according to World Health Organization classification [20], all tumors were classified as invasive ductal carcinoma.

§Breast tumor was graded according to the criteria described by Bloom and Richardson [21].

¶Estrogen receptor and progesterone receptor were determined by a dextran-coated charcoal method [22]. Tumors were considered hormone receptor–positive if they contained at least 10 fmol of specific binding sites per milligram of protein.

#During the mid-1980s, Halsted mastectomy or modified radical mastectomy preserving the pectoral muscles was widespread in Japan, although breast conservation surgery for breast cancer had become common in Western countries.

Results

Relation of ir-NE Content to Clinicopathological Factors

Table 1 shows the correlation between ir-NE content and the characteristics of the patients in this series. When ir-NE content was compared in terms of menstrual status, histologic type, histologic grade, vessel involvement, estrogen receptor, and progesterone receptor, no significant association was found between ir-NE content and any of these features. However, ir-NE content was significantly higher in tumors with a size of > 5.0 cm than in those with < 5.0 cm ($P < .002$). Similarly, ir-NE content was significantly higher in patients who were node-positive than in those who were node-negative.

Univariate Analysis

As expected, lymph node status, tumor size, histologic grade, vessel involvement, and adjuvant therapy were found to have a significant effect on disease-free survival when evaluated in a univariate analysis. When patient prognosis was analyzed in terms of the results of ir-NE, patients with breast cancer tissues containing a high concentration of ir-NE had a disease-free survival time significantly shorter than that in patients with a low content of ir-NE ($P = .0012$; Figure 1 and Table 2). In this analysis, the cutoff point of 9.0 μg/100 mg protein was used because our preliminary study of another 49 patients [17] revealed that this cutoff point could give a statistically significant separation for risk of relapse, according to the method of Tandon et al. [29]. This cutoff point identified 16.6% (52 of 313) of the patients as having high ir-NE levels in the present series.

Multivariate Analysis

To verify the independent nature of the prognostic value of ir-NE concentration, we used multivariate analysis. Cox regression analysis of overall survival, allowing for menstrual status, tumor size, lymph node status, histologic type, histologic grade, vessel involvement, estrogen receptor, progesterone receptor, type of surgery, adjuvant therapy, and ir-NE, showed that lymph node status is the single independent prognostic factor of disease-free survival ($P = .0012$; relative risk = 1.62; Table 2). To eliminate the effect of the inclusion of not so important variables into the model, we also performed stepwise regression analysis with a 5% significance level. Through a stepwise method, the model selected lymph node status ($P = .0004$; relative risk = 1.46) and ir-NE concentration ($P = .0013$; relative risk = 1.43) (Table 3). Menstrual status, tumor size, histologic type, histologic grade, vessel involvement, estrogen receptor, progesterone receptor, type of surgery, and adjuvant therapy were not independent prognostic factors.

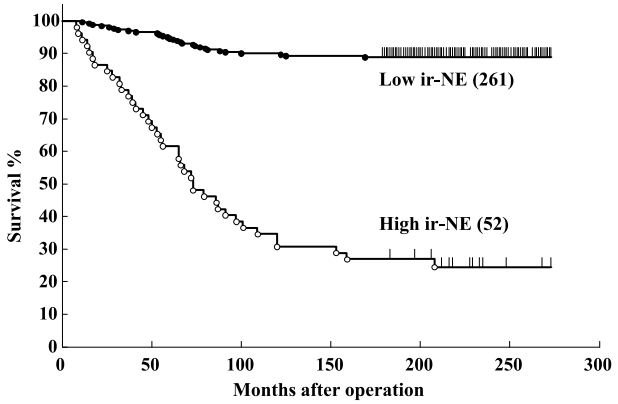

Figure 1. *Relapse-free survival curves in 313 patients with breast cancer in terms of ir-NE concentration in tumor extracts. The major statistical endpoint of this study was disease recurrence (distant recurrences only). The cutoff point between high and low enzyme levels is 9.0 μg/100 mg protein. Numbers in parentheses show the total number of patients per group.*

Table 2. Univariate and Cox Regression Analyses as Prognostic Factors for Relapse in Patients with Breast Cancer.

Variable	Univariate Analysis		Multivariate Analysis		Relative Risk
	P	*Z*	SE	*P*	
Independently associated with relapse					
Lymph node status (node-negative *vs* node-positive)	.0012	−1.68	0.54	.0001	1.62
Associated with relapse only when evaluated alone					
Tumor size (< 2.0 *vs* 2.0–5.0 *vs* > 5.0 cm)	.0431	0.46	0.32	.324	1.04
Histologic grade (I *vs* II *vs* III)	.0088	−0.74	0.39	.163	0.62
Vessel involvement (absent *vs* present)	.0315	−0.62	0.24	.112	1.51
Adjuvant therapy (endocrine *vs* chemotherapy *vs* both *vs* none)	.0048	0.53	0.33	.103	0.54
ir-NE (< 9.0 *vs* ≥ 9.0)	.0012	0.45	0.31	.062	1.50
Not associated with relapse					
Menstrual status (premenopause/perimenopause *vs* postmenopause)	.4226	−0.70	0.40	.504	1.57
Histologic type (papillotubular *vs* solid–tubular *vs* scirrhous *vs* other)	.1003	0.04	0.45	.754	0.17
Estrogen receptor (positive *vs* negative *vs* unknown)	.7314	−1.47	0.33	.214	1.08
Progesterone receptor (positive *vs* negative *vs* unknown)	.1571	−0.79	0.38	.133	1.52
Type of surgery (Halsted *vs* modified)	.9174	0.61	0.44	.417	0.81

Discussion

The purpose of identifying prognostic factors in breast cancer is to provide a sound basis for the rational management of the disease. Reliable predictors of cancer recurrence and death in patients with breast cancer may help to determine the selection of adjuvant chemotherapy or endocrine therapy. Classic prognostic factors, such as age, tumor size,

Table 3. Final Stepwise Regression Analysis.

Variable	*Z*	SE	*P*	Relative Risk
Lymph node status	−1.55	0.49	.0004	1.46
ir-NE	0.39	0.11	.0013	1.43

lymph node involvement, histologic grade, and hormone receptor status, assist in predicting the patient's outcome or response to treatment, but they are not entirely dependable. Several enzymes or biologic factors determined in the cytoplasm and organelles of tumor cells have been found to have prognostic value in human breast cancer [29,30].

In the present study, we have demonstrated that the ir-NE concentration in tumor extracts is an independent prognostic factor that clearly identifies patients at high risk and at low risk for the disease, indicating that this enzyme level can be added to the list of second-generation prognostic factors in human breast cancer [31–33]. NE is the only neutral protease that is able to degrade insoluble elastin [11,34]. NE can also hydrolyze other ECM proteins, including type IV collagens [35], fibronectins [36], and proteoglycans [37], and has been reported to potentiate the conversion of plasminogen to plasmin by urokinase-type plasminogen activator [38]—an enzyme that has been postulated to play a role in cancer spread [39]. Thus, tumor NE may play a pathologic role in facilitating cancer cell invasion and metastasis, either directly by the dissolution of the tumor matrix or indirectly through such a protease cascade. The results presented here—demonstrating that free-form (active form) NE, but not the α_1-PI–complexed form (inactive form), contributes to the prognostic value in human breast cancer—may support the above assumption.

The interactions between tumor and normal cells are complex events that occur continuously throughout the entire invasion process. A wide variability in the relative proportions of tumor and host cells has been observed at the zone of tumor invasion. Breast tumors also are heterogenous, with varying tumor cellularities and amounts of stroma. It is therefore possible that some of the NE proteins detected in this study that assayed tumor cytosols were extracted from infiltrating inflammatory cells and that this inflammatory cell involvement correlated with poor prognosis. Normal cells, such as neutrophils, fibroblasts, macrophages, and lymphocytes, all of which appear in the tumor invasion zone, may cooperate for the destruction of the host ECM. In fact, inflammatory cell infiltration has been reported to be associated with poor prognosis in human breast cancer [40].

In conclusion, tumor NE, whatever the cellular origin, may play an active role in the tumor progression that leads to metastasis in human breast cancer. The long-term follow-up results presented here, demonstrating that free-form NE is a strong and independent prognostic factor in human breast cancer, may support the above assumption.

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